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HISTOCHEMICAL DEMONSTRATION OF SIALOMUCIN IN HUMAN ECCRINE SWEAT GLANDS*

V. S. CONSTANTINE, M.D., M.S.† AND ROBERT W. MOWRY, M.D.‡

The presence of periodic acid-Schiff (PAS) positive diastase-resistant granules in the dark or mucoid cells of the human eccrine sweat gland secretory coil was described by Montagna and co-workers (1). In the same location were granules showing metachromatic basophilia with toluidine blue but which no longer stained after ribonuclease digestion. Formisano and Lobitz (2) described similar granules but reported that basophilia persisted despite prior digestion with ribonuclease. Lee (3) noted that the granules were stained by Steedman's Alcian blue 8 GS and that digestion with hyaluronidase had no effect. Munger (4) applied Mowry's colloidal iron stain in a combined light and electron microscopic study of the human eccrine sweat gland. The colloidal iron stain colored acidic material in the lumen of the glands, in the intercellular canaliculi and granules at the apices of the dark cells. These granules corresponded to the secretory granules seen by the electron microscope in the dark cells.

Hexosamine-containing mucoproteins were detected in thermally induced human sweat by Jirka and Kotas (5). More recently Pallavicini and co-workers (6) found that thermally induced human sweat also contained N-acetylneuraminic acid and hexoses.

The present study was undertaken to further elucidate the nature of the mucin in the secretory cells of the human eccrine sweat glands. Histochemical evidence obtained by us indicates that acidic carbohydrate-containing material in the secretory cells of human eccrine sweat glands is sialomucin; its general properties resemble those of sialomucins found in salivary glands, mammary glands and mucous glands of certain regions of the gastrointestinal tract. Possible significance of these findings to other fields of study will be discussed.

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* From the Department of Dermatology† and Department of Pathology‡ University of Alabama Medical Center, Birmingham, Alabama—35233.

MATERIALS AND METHODS

Specimens of skin were obtained from the abdominal incisions and back of the necks of 12 adults at autopsy. In addition, seven specimens of skin from different areas of the body were removed from the margins of widely excised skin tumors. The specimens were fixed in 10% neutral buffered formalin (7), embedded in paraffin and cut at 4 to 6 micra. None of the studies showed any difference between autopsy and surgical material.

A variety of histochemical precedures were applied to each specimen. The colloidal iron and Alcian blue stains for acidic carbohydrates were performed as described by Mowry (8).

Sulfated polysaccharides were tested for by two methods, *viz.*, high iron-diamine stain and the low pH Alcian blue stain. (Basophilia of complex carbohydrates can result from sulfate groups alone, carboxyls alone or a mixture). Alcian blue used in 0.5 N hydrochloric acid is said to stain only sulfated carbohydrates (9, 10). The importance of care in rinsing after staining was noted by Lev and Spicer (11) who blotted sections dry and then dehydrated directly without washing in order to prevent staining of carboxyl groups observed to occur during washing.

Mowry (12) has found that staining at such low pH seems restricted to polyanions with sulfate groups but that a more convenient procedure can be used. Sections were stained for 2 hours in 0.25% Alcian blue 8 GX in 0.5 N hydrochloric acid followed by three rinses in 0.5 N hydrochloric acid, each for 3 minutes. Then sections were rinsed in three changes of 0.3% sodium carbonate for 5 minutes each. Afterwards sections can be washed and counterstained as desired without fear of coloring polyanions having only carboxyl groups. Sections were counter-stained in hematoxylin as in Mowry's revised Alcian blue-hematoxylin stain (8). This modification has the advantages of allowing the use of counterstains and dispensing with the messiness encountered when sections are blotted directly from the dye solution.

The high iron-diamine stain was performed as directed by Spicer (13) except that no Alcian blue counterstain was used.

For the identification of sialomucin, neuraminidase digestion was used. This enzyme was first used in histochemistry by Spicer and Warren (14). Sections of skin and some tissues known to contain sialomucin were incubated for 24 hours at 37° C in

a solution of *Vibrio cholerae* neuraminidase[1] containing 0.05 M pH 5.5 sodium acetate-acetic acid buffer, 0.9% sodium chloride and 0.1% calcium chloride. As a control against non-specific solution, a set of duplicate sections were treated also for 24 hours in the buffer alone. The two sets of sections were deparaffinized and hydrated. The area around the section was blotted dry. With a glass-marking wax pencil a ring was drawn around the section forming a shallow well. These slides were placed inside a Petri dish atop moistened filter paper. The appropriate solution was applied to the section inside the wax mark and the dish sealed by a thin rim of vaseline between the rim and the lid. The dishes were kept at 37° C. for 24 hours. No evaporation of enzyme solution or the buffer occurred. Further addition of enzyme was unnecessary. After incubation all slides were washed five minutes in running water. Both the enzyme-treated and the control set exposed only to buffer were stained by Mowry's revised colloidal iron method and counterstained with Harris' hematoxylin and the Van Gieson stain.

RESULTS

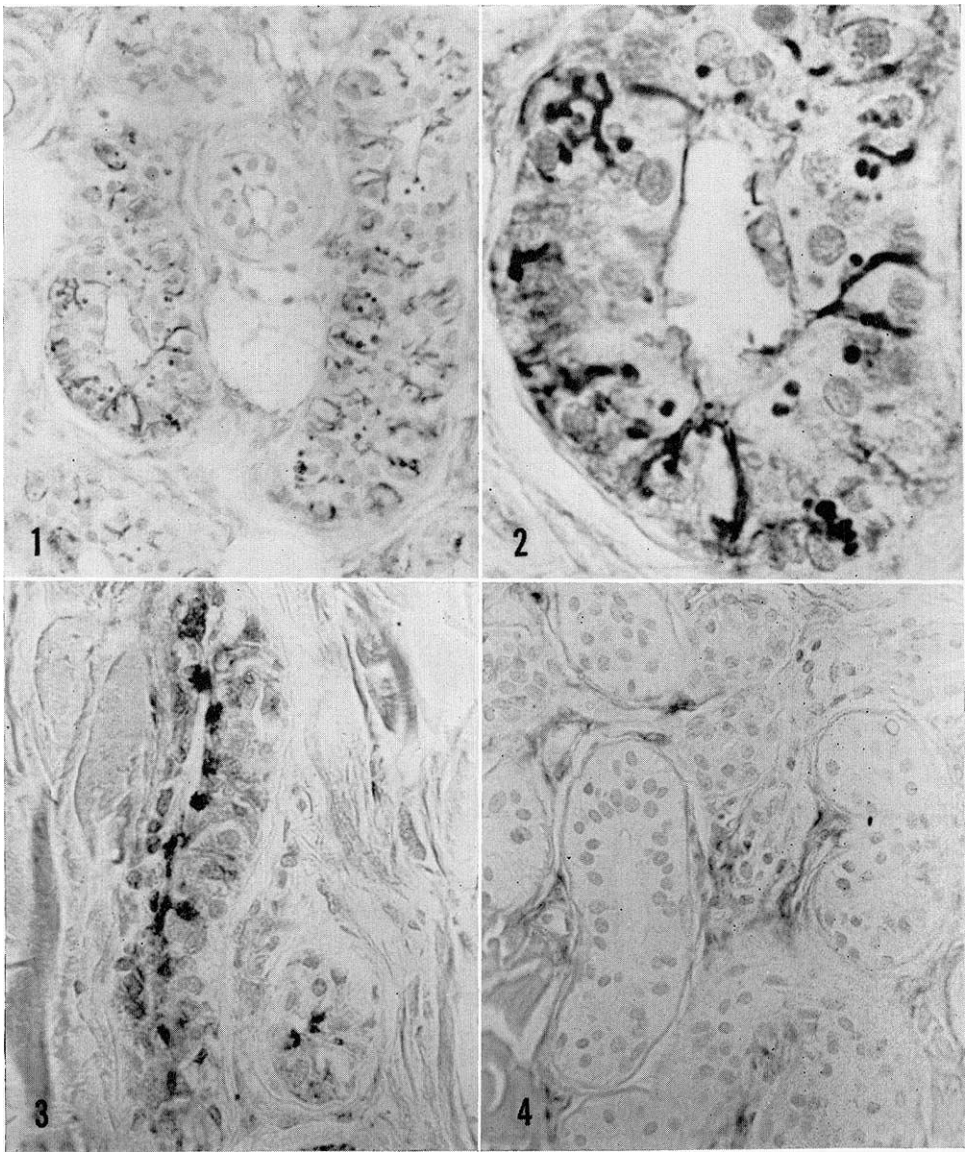
With Alcian Blue 8 GX and with the Colloidal Iron Stain.—All but one of the skin specimens showed eccrine sweat glands that contained varying amounts of acidic material, presumably acidic carbohydrate. The acidic material occurred in the secretory portion of the glands. Lumens of sweat ducts were devoid of such material, possibly dissolved during histologic processing. The exact distribution of acidic material within the secretory portions varied in different specimens. The amounts varied also, but were usually moderate to slight. The most typical appearance was filling of intercellular canaliculi between clear cells by acidic material that often was continuous with a coating of similar material on the luminal surface of secretory cells. Rarely, glandular lumens were filled completely with acidic material; solution of secretion here also may occur during histologic processing. In some specimens the apical cytoplasms of secretory cells were more or less filled with acidic material. In one specimen there was fairly diffuse though slight coloration of the general cytoplasm of secretory cells. In the other specimens diffuse cytoplasmic staining seldom was seen.

[1] The buffered enzyme solution containing 100 units of *Vibrio cholerae* neuraminidase per ml was purchased from Behringwerke AG, Marburg-Lahn, West Germany. One unit, as defined by the supplier, is that amount of enzyme which will liberate 1 microgram of acetylneuraminic acid in 15 minutes at 37° C from a standard glycopeptide solution.

The acidic material was colored more strongly by the colloidal iron stain than by Alcian blue. Demonstration of the intercellular canaliculi was in general a striking feature of most sections stained by the colloidal iron method. These canaliculi were colored by Alcian blue but less distinctly, as noted by Munger (4). Duplicate sections stained for naturally-occurring iron, a necessary safeguard in the use of the colloidal iron stain, showed no coloration of eccrine sweat glands. Coloration by the colloidal iron reaction and by Alcian blue in paraffin sections signifies usually complex carbohydrates with acidic groups, either carboxyl groups, sulfate or both (8). While each of these methods colors carboxyl-rich polyanions especially well, they also color certain sulfated polyanions. Further analysis required additional evidence.

With the Low pH Alcian Blue and with the High Iron-diamine Procedures for the Detection of Sulfated Polyanions.—Complete absence of coloration in the secretory cells of the eccrine sweat glands was observed in every specimen tested. Excellent coloration was obtained in mast cells, believed to contain heparin which is rich in sulfate groups.

The Effect of Neuraminidase Digestion on the Subsequent Stainability of Eccrine Sweat Glands with the Colloidal Iron Stain.—Sweat glands in sections exposed for 24 hours to buffer alone followed by the colloidal iron stain showed only a very slight decrease in the amount of acidic material, due probably to either partial solution or hydrolysis. Despite this, sweat glands in each control section showed definite coloration typically of the intercellular canaliculi or coating the lumen of the gland or in the apical cytoplasms of certain cells presumably so-called mucoid or dark cells (Figures 1–3). Different glands in the same specimen varied, as did individual cells. In contrast, there was *complete absence* of acidic material in sweat glands of every specimen incubated with neuraminidase before performance of the colloidal iron stain (Figure 4). Other acidic materials in the same sections, for example, presumed mucopolysaccharides in the connective tissues and blood vessels were largely unaffected by neuraminidase. Sections of pig submaxillary gland and other tissues known to contain sialomucins were similarly tested; these



All preparations shown were stained with the colloidal iron stain and counterstained with Harris' hematoxylin and Van Gieson's picrofuchsin. Photomicrographs were taken using a Kodak red filter (A-25) for the purpose of enhancing contrast between sites of colloidal iron staining and those features colored by the counterstains. Material stained by colloidal iron appears black in the photographs.

FIG. 1. Secretory coil of eccrine sweat gland containing presumed sialomucin colored by the colloidal iron stain. This is a control preparation incubated for 24 hours at 37°C. in pH 5.5 acetate buffer containing calcium but without neuraminidase, × 300.

FIG. 2. This is a higher magnification showing part of the secretory coil seen on the left side of Figure 1. Acidic material appearing black in the photograph outlined sharply many but not all of the intercellular canaliculi. Much less acidic material coats part of the luminal surface of some cells. Rounded masses of acidic material seeming to be in the cytoplasms of some cells may represent cross sections of intercellular canaliculi in adjacent cells, × 900.

FIG. 3. Secretory portion of sweat gland in another specimen of skin treated in the same way as in Figure 1. Shown is acidic material in the cytoplasms of many secretory cells, concentrated in the apices. Here secretory canals are not evident as with the previous figures, × 300.

FIG. 4. This is a typical appearance of the results obtained after neuraminidase digestion for 24 hours. There is complete absence of colloidal iron stained material within sweat glands. Some foci of blackening in the interstitial tissue is the result of colloidal iron staining presumably of acid mucopolysaccharides unaffected by neuraminidase, × 300.

also showed complete absence of stainability in the appropriate sites that were colored in duplicate sections stained by colloidal iron after exposure to buffer alone.

DISCUSSION

Acidic material presumed to be complex carbohydrate was present in human eccrine sweat glands in nearly all specimens of skin examined. The amount was not great. Others have described the presence of complex carbohydrates in the secretory cells of eccrine sweat glands (1–4). This acidic material was colored more strongly and more definitely by the colloidal iron stain than by Alcian blue which may explain why previous workers found less frequent Alcian blue staining in the sweat glands (15, 16). Not all procedures for the use of Alcian blue are equally effective (8).

Our experiences support those of Munger (4) who described colloidal iron-positive material within the intercellular canaliculi as well as in the apical cytoplasms of dark cells. Restriction of the acidic material to secretory portions of the eccrine sweat glands suggests origin of the acidic material there. Admittedly, secretion in the sweat ducts may be too soluble to be retained by aqueous formalin used here. The amount of acidic material varies among different cells, different glands and among the different specimens examined. Such differences in the content of acidic carbohydrates may well reflect functional differences or other influences that are not yet determined. We agree with Munger (4) that, when present, the staining of acidic material in intercellular canaliculi by colloidal iron provides an elegant demonstration of these structures. Occurrence of the acidic material in the canaliculi suggests but does not prove its origin from clear cells. It is also possible, though we think less likely, that the acidic carbohydrates might reach the secretory canals by reflux from the main lumen of the gland. Occasionally gland lumens were filled largely with acidic material. Its absence in most gland lumina suggests that it may have dissolved during fixation. Whether the inconstant presence of acidic material, both in intercellular canaliculi and in lumina, is the result of incomplete retention of the material or signifies varying functional states of the gland is not yet clear.

The finding of acidic carbohydrate in the apical cytoplasm of secretory cells of sweat glands was infrequently observed. If these are the dark cells described by others, then it may be that both dark and clear cells are capable of forming acidic carbohydrates. While the cytoplasm of clear cells generally contains no acidic carbohydrate, intercellular canaliculi cut in cross sections might simulate such an appearance to the unwary. It is possible also that other forms of fixation and tissue handling designed to retain more completely complex carbohydrates that might dissolve partly in water or other fluids may lead to a more constant distribution and more complete retention of the acidic material in sweat glands.

Greater sensitivity of the colloidal iron stain compared with that of Alcian blue in certain other applications has been noted by Mowry (17). In general, polyanions containing only carboxyl groups are demonstrated better by each of these methods than by toluidine metachromasia. Greater staining by the colloidal iron reaction compared with Alcian blue is more typical of polyanions rich in carboxyl groups than for sulfated polyanions. Negative results with two different procedures used to test for sulfate groups strengthens the likelihood that the acidic carbohydrates of sweat glands contain mainly or solely carboxyl groups. The finding of colloidal iron-positive material within the intercellular canaliculi suggests the possibility of its use to clarify its exact location by electron microscopy. Colloidal iron is electron dense and has been used in some other applications for the localization of acidic carbohydrates in relation to ultrastructure (18, 19).

Neuraminidase, also known as sialidase, was first introduced into histochemistry by Spicer and Warren (14). These workers showed that in tissues of rodents a number of acidic mucins lacking attributes of sulfated polyanions were no longer stainable after treatment with neuraminidase obtained from various sources. So-called sialomucins have in common sialic acid whose acidity depends on carboxyl groups. Identified first in submaxillary gland mucin, sialic acid-containing glycoproteins and glycopolypeptides are now known to occur widely in mammalian tissues, typically in epithelial secretions, but also in some connective tissues including dermal connective tissue (20).

The sialomucins originating in different tis-

sues may differ in some structural details but have in common sialic acid in the prosthetic part of the carbohydrate moiety. Although physically similar and similar also in some staining reactions, sialomucins differ chemically from acid mucopolysaccharides of connective tissues and should not be referred to as such. Sialomucins have been identified in tissues of all vertebrates so far tested (21). It will not be surprising if eccrine sweat glands in other species prove also to contain sialomucin. As a generality, epithelial mucins that are acidic but non-sulfated should be considered as possible sialomucins until proven otherwise. Presumptive identification of the acidic carbohydrate in human eccrine sweat glands as sialomucin illustrates anew the value of neuraminidases in the histochemical study of complex carbohydrates, especially mucinous secretions.

Not all sialomucins are susceptible to neuraminidase and this complicates the interpretation of histochemical results when a particular acidic mucin proves resistant. Such resistance to the action of neuraminidases might result from steric differences in the attachment of sialic acid to the rest of the molecule (22–26). Abolition of basophilia by neuraminidase is therefore far more meaningful than lack of an effect. Absence of all stainability of human eccrine sweat glands with colloidal iron after neuraminidase treatment is presumptive evidence that the secretory cells and their secretion contain sialomucin.

The histochemical results support observations made by others using chemical methods (6). These workers reported analyses indicating that sulfate-containing material was absent and that N-acetylneuraminic acid was present.

Neuraminidase preparations so far available for general use are not so highly purified that short incubation periods can yet be used. It is hoped that with purer preparations or increased knowledge of the conditions promoting neuraminidase activity, it will be possible to use shorter incubation periods in histochemical applications. Use of shorter incubation periods lessens the possibility of error due to the action of any contaminating enzymes, *e.g.*, proteases that might also affect the substrate.

Knowledge that human eccrine sweat glands contain sialomucin suggests its possible value as a parameter of function and as a test object in future histochemical studies, for example, its comparison with other sialomucins. Among sialomucins so far identified histochemically in human tissues are those of certain regions of the gastrointestinal tract, respiratory tract and salivary glands (27). We, as well as others (28), have identified neuraminidase-labile sialomucins in ductal secretion of breast studied in tissue sections and in mucinous carcinomas of breast. It has also been found that acidic material in epithelial cells of renal glomeruli in humans and other mammalian species contain acidic material digestable with neuraminidase and presumed to be sialomucin (29). Abnormality of sweat glands in fibrocystic disease of the pancreas relates better to other features of the disease in the light of the apparent sialomucin-containing secretion with histochemical properties similar to those occurring in many other epithelial sites.

If sweat glands of other species prove to contain sialomucins, analytical studies of possible variations with age, functional states, hormones and pharmacologic agents will be much facilitated. Clear demonstration of intercellular canaliculi in secretory cells of many eccrine sweat glands by colloidal iron whenever acidic carbohydrates are present facilitates the demonstration of these unusual structures. Intercellular canaliculi are said to be well demonstrated also in sections tested for alkaline phosphatase activity (30). Tissues to be studied for alkaline phosphatase activity require specialized fixation which is not required for use of the colloidal iron stain. Simultaneous demonstration of both sialomucin content and alkaline phosphatase in the same preparation should be feasible and may prove of interest in assessing functional activities of the gland.

SUMMARY

Eccrine sweat glands in paraffin sections of human skin fixed in neutral buffered formalin nearly always contained acidic carbohydrate detected by the colloidal iron stain, and to a less extent by Alcian blue 8 GX. Additional histochemical procedures designed to detect sulfated carbohydrates showed none. The acidic carbohydrate demonstrated in eccrine sweat glands was presumed to contain only carboxyl groups. As incubation of tissue sections in neuraminidase abolished stainability of the acidic carbohydrate

with colloidal iron, the material removed was considered to be a sialomucin. To the best of our knowledge, the identification of the acidic mucin in human eccrine sweat glands as sialomucin has not been reported previously.

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